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Low-Concentration Kinetics of Atmospheric CH_4 Oxidation in Soil and Mechanism of NH_4^+ Inhibition

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NH_4^+ inhibition kinetics for CH_4 oxidation were examined at near-atmospheric CH_4 concentrations in three upland forest soils. Whether NH_4^+-independent salt effects could be neutralized by adding nonammoniacal salts to control samples in lieu of deionized water was also investigated. Because the levels of exchangeable endogenous NH_4^+ were very low in the three soils, desorption of endogenous NH_4^+ was not a significant factor in this study. The $K_{m(app)}$ values for water-treated controls were 9.8, 22, and 57 nM for temperate pine, temperate hardwood, and birch taiga soils, respectively. At CH_4 concentrations of ≤15 μl liter^{-1}, oxidation followed first-order kinetics in the fine-textured taiga soil, whereas the coarse-textured temperate soils exhibited Michaelis-Menten kinetics. Compared to water controls, the $K_{m(app)}$ values in the temperate soils increased in the presence of NH_4^+ salts, whereas the $V_{max(app)}$ values decreased substantially, indicating that there was a mixture of competitive and noncompetitive inhibition mechanisms for whole NH_4^+ salts. Compared to the corresponding K^+ salt controls, the $K_{m(app)}$ values for NH_4^+ salts increased substantially, whereas the $V_{max(app)}$ values remained virtually unchanged, indicating that NH_4^+ acted by competitive inhibition. Nonammoniacal salts caused inhibition to increase with increasing CH_4 concentrations in all three soils. In the birch taiga soil, this trend occurred with both NH_4^+ and K^+ salts, and the slope of the increase was not affected by the addition of NH_4^+. Hence, the increase in inhibition resulted from an NH_4^+-independent mechanism. These results show that NH_4^+ inhibition of atmospheric CH_4 oxidation resulted from enzymatic substrate competition and that additional inhibition that was not competitive resulted from a general salt effect that was independent of NH_4^+.

Atmospheric CH_4 contributes substantially to the greenhouse effect, and the concentration of atmospheric CH_4 has increased dramatically in the past century because of human activity associated with agriculture, land use changes, and industry (34, 35). Bacterial oxidation of atmospheric CH_4 in well-drained soils is an important regulator of atmospheric CH_4 concentration, yet the organisms responsible remain unidentified and the physiology of the process is poorly understood (9, 35, 36). Although soil CH_4 consumption is inhibited by a wide variety of anthropogenic disturbances, such as agriculture, N deposition, and forestry (12, 17, 22, 23, 32, 43, 44), predictable inhibition patterns have failed to emerge, which has made it difficult to predict the effects of disturbance on soil CH_4 flux in various ecosystems. The most commonly reported disturbance effect is that of NH_4^+ fertilizers, which can suppress soil CH_4 consumption by up to 70% (1, 8, 10, 17, 22, 32, 33, 37, 38, 43). In the field, inhibition may occur immediately following fertilization, may be delayed for months to years, or may never occur despite years of chronic fertilization (9, 17). This variety of responses may stem at least in part from the distribution of physiologically diverse methane oxidizer populations across sites (17, 18, 20).

Of the various NH_4^+ inhibition patterns, immediate inhibition is the best documented. As in field studies, however, physiological laboratory studies have produced variable results, suggesting that there may be multiple inhibition mechanisms (15, 17, 26–28, 36, 39). Physicochemical similarities between CH_4 and NH_3 may permit these two compounds to compete for enzyme active sites so that fortuitous NH_3 oxidation competitively inhibits CH_4 oxidation (38). Although this mechanism has been demonstrated to occur in pure cultures of methanotrophic bacteria (6) and in a CH_4-producing agricultural soil (15), it has not been demonstrated to occur in well-drained, nonagricultural mineral soils, which comprise the dominant terrestrial sink for atmospheric CH_4 (14, 38, 45), nor has it been demonstrated to occur at near-atmospheric CH_4 concentrations. In many cases, the kinetics of immediate NH_4^+ inhibition in soil cannot be reconciled easily with substrate competition (15, 16, 26–28, 39). An alternative mechanism has been proposed, whereby the toxicity of NO_2^- or NH_2OH produced by fortuitous NH_4^+ oxidation suppresses methanotrophic activity (26, 27, 39). Hence, multiple inhibition mechanisms may be involved, and these mechanisms may vary with the physiology of different CH_4 oxidizer populations (17).

Two physiologically distinct communities of CH_4 oxidizers apparently exist in soil. One group, generally associated with atmospheric CH_4 consumption, exhibits an extremely high affinity for CH_4. Representatives of this group have yet to be cultivated or otherwise identified (9). The second group exhibits a much lower affinity for CH_4 and is generally associated with common methanotrophs, such as those that have been studied in pure culture for many years (2, 9). In upland mineral soils, only high-affinity activity is usually detectable without artificial enrichment with high CH_4 concentrations in the laboratory. However, the only prior study in which kinetic constants for NH_4^+ inhibition of soil CH_4 oxidation were reported was conducted in a periodically moist, organic matter-rich agricultural soil with demonstrable methanogenesis (15, 16). Such a soil potentially harbors a rich community of CH_4 oxidizers representing a continuum from low-affinity organisms to high-affinity organisms. Although this important investigation

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TABLE 1. Sampling sites and characteristics of the soils examined in this study

Ecosystem type (dominant plant)	Location	Soil texture	Concn of extractable NH_4^+ (mg of N kg of dry soil^{-1})[a]	Water-holding capacity (g of H_2O g of dry soil^{-1})
Birch taiga (*Betula papyrifera*)	University of Alaska, Fairbanks	Fine silt	0.11 (0.014)[b]	0.62
Temperate hardwood (*Quercus velutina*)	Harvard Forest, Petersham, Mass.	Sandy loam	6.98 (2.91)[c]	0.84
Temperate pine (*Pinus resinosa*)	Harvard Forest, Petersham, Mass.	Sandy loam	7.83 (2.49)[c]	0.73

[a] concentration of NH_4^+ N in the upper 10 cm of the mineral horizon, which includes the zone of maximum CH_4 oxidation. The values in parentheses are standard deviations.
[b] Data from this study. The concentration of extractable NH_4^+ was determined as described previously (17).
[c] Data from A. Magill (29a). See reference 30 for more information.

demonstrated that NH_4^+ inhibits CH_4 oxidation via enzymatic substrate competition in an agricultural humisol, it is unclear to what extent its results apply to well-drained mineral soils lacking endogenous CH_4 sources. Physiological studies of soil CH_4 oxidation typically derive kinetic constants from oxidation rates at CH_4 concentrations ranging from atmospheric levels (~1.7 μl liter^{-1}) to $\gg K_m$ for high-affinity CH_4 oxidizers. Even in soil in which only high-affinity organisms are active, the CH_4-oxidizing enzyme(s) could respond differently to NH_4^+ at high CH_4 concentrations than at near-atmospheric concentrations (15, 39). Thus, to study NH_4^+ inhibition of high-affinity CH_4 oxidizers per se, it would be preferable to examine inhibition kinetics at near-atmospheric CH_4 concentrations in a soil with no apparent endogenous CH_4 source.

A common shortcoming of NH_4^+ inhibition studies, regardless of the organisms involved, has been a lack of attention to nonammoniacal salt effects despite numerous reports of substantial inhibition by such salts (1, 10, 15, 17, 24). King and Schnell (28) examined the effects of several Cl^- and SO_4^{2-} salts and concluded that nonammoniacal salts indirectly inhibit CH_4 oxidation by desorbing endogenous NH_4^+ from cation exchange sites in the soil, which then directly inhibit CH_4 oxidation. Many N-limited soils, however, have extremely low concentrations of exchangeable NH_4^+, yet are substantially inhibited by nonammoniacal salts (17), suggesting that these salts have NH_4^+-independent effects on atmospheric CH_4 oxidizers. Additional mechanisms may alter inhibition kinetics, thus hindering the diagnosis of NH_4^+-specific inhibition.

With the limitations described above in mind, we used a simple steady-state kinetics approach to assess the mechanism of NH_4^+ inhibition of CH_4 oxidation at near-atmospheric concentrations (1.8 to 15 μl liter^{-1}) in three well-drained, N-limited forest soils that lack known endogenous CH_4 sources. In addition, we examined the effects of nonammoniacal salts in parallel samples to judge the utility of these salts as experimental controls for neutralizing NH_4^+-independent salt effects.

MATERIALS AND METHODS

Field sites. We studied soils from two temperate forests and one taiga forest, the major characteristics of which are listed in Table 1. The two temperate soils were from the Harvard Forest Long-Term Ecological Research site in western Massachusetts (29), where fertilizer inhibition of atmospheric CH_4 consumption was first observed (43). The sites and their biogeochemical cycles have been described in detail previously (7, 8, 30). The taiga site is approximately 120 years postburn, and the understory is dominated by *Rosa acicularis* and *Equisetum* spp. The mineral soil consists of a uniform layer of silty glacial loess. The pedology, ecology, and biogeochemistry of this site are similar to the pedology, ecology, and biogeochemistry of nearby sites that have been described previously (17). All three sites are well drained and have never been observed to produce CH_4 (7, 8, 19).

Soil processing and bioassays. All experiments were performed at the University of Alaska, Fairbanks. At each site, soil was collected in bulk from the upper 10 cm of the mineral soil, which included the zone of maximum CH_4 oxidation, and stored in perforated plastic bags for transport to the laboratory. The soil was homogenized by sieving it through a 4-mm-mesh screen. The water-holding capacity of each soil type was determined as described previously (18), and the moisture was adjusted so that the final water content was 30 to 35% of the water-holding capacity (Table 1) after the final treatment with deionized water or salt solutions. This moisture level was determined previously to be optimal for atmospheric CH_4 consumption in a wide variety of soils (18). Samples were treated with deionized water, K_2SO_4, $(NH_4)_2SO_4$, Na_2SO_4 (taiga soil only), KCl, or NH_4Cl. Each salt solution was added to a single bulk sample (0.1 ml g of dry soil^{-1}), which was then mixed thoroughly and subdivided into individual samples. The salts were added at a rate of 5.6 μmol of cations per g of dry soil, so that all of the salts were equinormal with respect to cations. The resulting NH_4^+ additions were equivalent to 75 mg of N per kg of dry soil, which matched the treatments used in previous experiments (17). This amount of NH_4^+ was intended to overwhelm the endogenous soil N (Table 1) yet remain within the range of soil NH_4^+ concentrations reported for forest soils with various land use histories (13, 31, 42).

For each treatment, 12 subsamples (10 g of dry soil) were placed in 70-ml serum vials sealed with butyl rubber septa and allowed to equilibrate overnight. The following morning the vials were equilibrated with laboratory air (~1.8 μl of CH_4 liter^{-1}) and sealed, and their headspace CH_4 concentrations were adjusted by injecting appropriate volumes of 1% CH_4 premixed with air (Scott Specialty Gases, Plumsteadville, Pa.); the headspace CH_4 concentrations tested were approximately 1.8 (no CH_4 added), 5, 10, and 15 μl liter^{-1}. Three replicates for each treatment at each CH_4 concentration were prepared. For the temperate soils, a single 2-h CH_4 oxidation assay was carried out with the headspace CH_4 concentration measured at the beginning and the end of the assay. The resulting consumption rate ($d[CH_4]/dt$) was paired with the corresponding midpoint CH_4 concentration in order to obtain a plot of oxidation rate versus CH_4 concentration (Fig. 1b and c). For the birch taiga soil, a modified procedure was used because oxidation was 2 orders of magnitude slower in this soil than in the temperate soils (Table 2). On the first day of the experiment, a 3.3-h assay was carried out with the headspace CH_4 concentration measured at the beginning and the end of the assay. The samples were kept sealed and were allowed to consume CH_4 overnight, and the 3.3-h assay was repeated on the second day and again on the third day. Identical assays were then repeated every 48 h until either a threshold concentration was established or through the ninth day, whichever occurred first. As with the temperate soils, the rate ($d[CH_4]/dt$) from each 3.3-h assay was plotted against the corresponding midpoint CH_4 concentration in order to obtain a plot of oxidation rate versus CH_4 concentration (Fig. 1a). CH_4 was analyzed by gas chromatography as described previously (5, 17, 18).

Because Cl^- inhibited CH_4 oxidation much more than did SO_4^{2-}, the effects of Cl^- and SO_4^{2-} salts on general microbial respiration in the birch taiga soil were examined. The amount of CO_2 that accumulated was determined by measuring headspace CO_2 concentrations, which never exceeded 2%, at the beginning and end of a 1-week incubation period. The amount of CO_2 that accumulated in each salt treatment was compared to the amount of CO_2 in water-treated controls. CO_2 was analyzed by gas chromatography as described previously (5, 18).

Statistical analyses and calculations. The effects of the salt treatments and CH_4 concentration on oxidation rates in each soil were analyzed by analysis of covariance by using treatment as the independent factor and the initial CH_4 concentration as a covariate; Bonferroni contrasts were used in multiple comparisons. Because the incubation times were the same for all treatments in a given soil, the treatments with higher oxidation rates consumed more substrate than the treatments with lower oxidation rates. For regression analyses, therefore, the oxidation rate from an individual assay was paired with the corresponding midpoint CH_4 concentration (Fig. 1) rather than the initial concentration. This standard technique normalizes consumption rates for unequal substrate concentrations among treatments and also minimizes the deviation from standard Michaelis kinetics that can result from substrate depletion (41). First-order kinetics were modeled by linear regression, and the rate constants were esti-

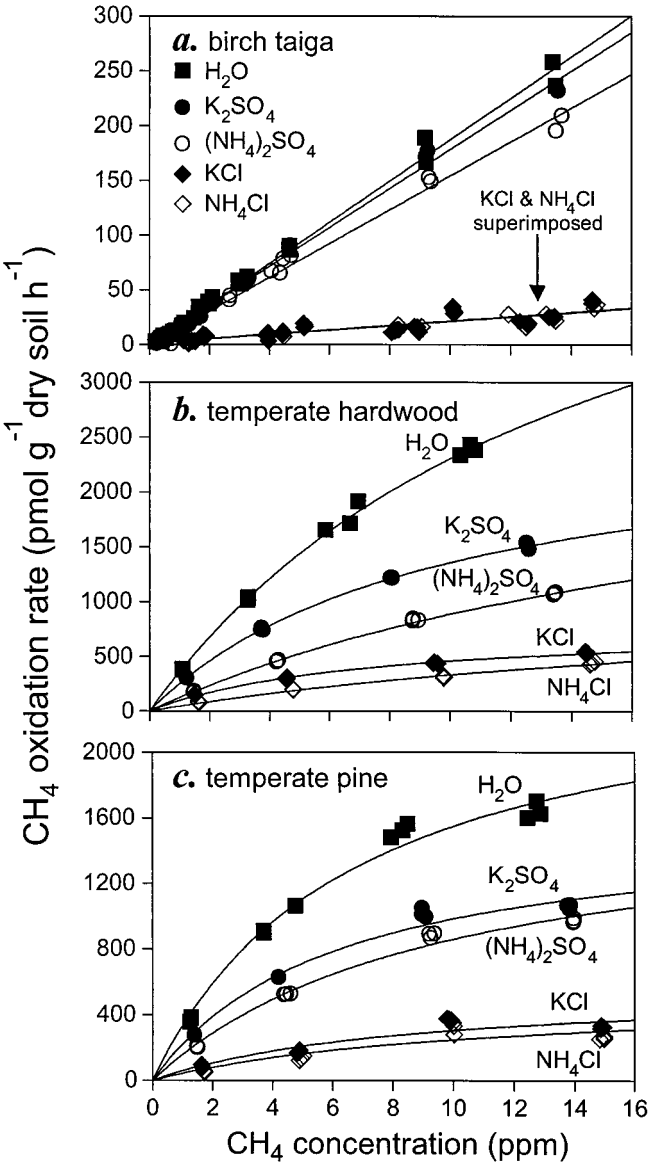

FIG. 1. CH_4 oxidation kinetics in three upland forest soils, a birch taiga soil (a), a temperate hardwood soil (b), and a temperate pine soil (c). The data for the birch taiga soil are shown with linear regression lines, whereas the data for the temperate soils are shown with nonlinear regression curves fit to the Michaelis-Menten equation. Each point represents a single rate measurement.

mated from the slope of the regression line, with both variables expressed in picomoles. Michaelis constants were obtained from a least-squares nonlinear regression fit of the data to the Michaelis-Menten equation. For treatments exhibiting Michaelis kinetics, pseudo-first-order rate constants were calculated as V_{max}/K_m, with both constants expressed in picomoles. Relative inhibition was calculated for each treatment as follows: relative inhibition = $(1 - k_2/k_1) \times 100$, where k_1 is the first-order (or pseudo-first-order) rate constant for the control sample and k_2 is the first-order (or pseudo-first-order) rate constant for the treated sample.

Examining the relationship between relative inhibition and CH_4 concentration requires calculating inhibition ratios for two treatments at specific CH_4 concentrations. In doing this, care must be taken not to compare rates derived from substantially different midpoint CH_4 concentrations, even when the initial concentration is the same for all treatments. This problem arises when a faster sample consumes substantially more substrate than a slower sample, resulting in a disparity between midpoint CH_4 concentrations in the two assays. For this reason, the method used to calculate relative inhibition at specific CH_4 concentrations varied according to the relative rates among treatments and the type of kinetics involved for each soil. In the birch taiga soil, which displayed first-order kinetics, inhibition ratios were calculated directly from the rates measured in the experiments on the first day of incubation. Because the oxidation rates were very low in this soil, the differences in midpoint CH_4 concentrations among the treatments were trivial. The inhibition by each salt compared to the deionized water control was calculated for each of the initial CH_4 concentrations (~1.8, 5, 10, and 15 μl liter^{-1}) and plotted against the midpoint CH_4 concentration occurring in the control (Fig. 2a). In the temperate soils, which displayed Michaelis kinetics, the rates were high, and the midpoint CH_4 concentrations varied among the treatments. Hence, estimated inhibition ratios were calculated by entering four different CH_4 concentrations (1.8, 5, 10, and 15 μl liter^{-1}) into the regression equation obtained for each treatment. The calculated oxidation rates at each concentration were then used to calculate inhibition ratios for each salt compared to the water control. Each ratio was then plotted against the CH_4 concentration from which it was derived (Fig. 2b and c). The slope of the relationship between relative inhibition and CH_4 concentration was estimated by linear regression.

RESULTS

Birch taiga soil. In the birch taiga soil, the CH_4 oxidation kinetics at concentrations of ≤15 μl liter^{-1} were approximately first order ($R^2 > 0.99$ except for Cl^- salts) for all treatments, but the rate constants varied among treatments (Fig. 1a; Table 2). Analyses at higher CH_4 concentrations (10 to 800 μl liter^{-1}) (data not shown) yielded a $K_{m(app)}$ for oxidation in this soil of 39 μl liter^{-1} (57 nM in solution), which is typical for upland soils (2, 3, 36, 46, 47). Neither K_2SO_4 nor Na_2SO_4 significantly inhibited CH_4 oxidation compared to deionized water ($P = 0.78$), and the curves for K_2SO_4 and Na_2SO_4 were indistinguishable ($P = 0.91$) (Na_2SO_4 data not shown). Specific NH_4^+ inhibition, calculated using K_2SO_4 as the control, was relatively weak (19%) but was statistically significant ($P = 0.04$). Both KCl and NH_4Cl inhibited CH_4 oxidation severely (~90%; slopes were significantly different from zero at $P < 0.01$) (Fig. 1a). Unlike the comparison of K_2SO_4 and $(NH_4)_2SO_4$, the effects of KCl and NH_4Cl were indistinguishable ($P = 0.84$) (Fig. 1a). All four salts caused relative inhibition to increase as CH_4 concentration increased (Fig. 2a). The slopes of the increases were similar for all salts regardless of which cation was added and regardless of the final soil NH_4^+ concentration. All salts inhibited total microbial respiration, but like CH_4 oxidation, CO_2 production was more sensitive to Cl^- salts than to SO_4^{2-} salts; the relative inhibition was ~18% for both K_2SO_4 and $(NH_4)_2SO_4$, whereas it was 22 to 25% for KCl and NH_4Cl.

Temperate forest soils. The relative inhibition patterns for the various salts in the temperate hardwood and pine forest soils were similar to the patterns in the birch taiga soil, except that CH_4 oxidation conformed well to Michaelis-Menten kinetics ($R^2 > 0.98$ in most cases) (Fig. 1b and c; Table 2). With minor differences in magnitude, the pine soil exhibited the same patterns as the hardwood soil. As in the birch taiga soil, the Cl^- salts were the most inhibitory salts, followed by $(NH_4)_2SO_4$ and then K_2SO_4. K_2SO_4 inhibition and specific NH_4^+ inhibition (relative to K^+) were stronger in the temperate soils than in the taiga soil; the levels of specific NH_4^+ inhibition in the temperate hardwood and pine soils were 54 and 34%, respectively (Table 2). As in the birch taiga soil, inhibition of CH_4 oxidation increased with the CH_4 concentration when K^+ salts were added. Unlike the taiga soil, however, inhibition in the temperate soils decreased as CH_4 concentration increased when NH_4^+ salts were added (Fig. 2b) (pine forest results not shown). When specific NH_4^+ inhibition was calculated using K^+ salts as controls, inhibition decreased sharply from ~50% in the presence of 1.8 μl of CH_4 liter^{-1} to ~20 to 30% in the presence of 15 μl of CH_4 liter^{-1} in the temperate hardwood soil (Fig. 2c).

The Michaelis parameters K_m and V_{max} exhibited similar patterns of responses to the various treatments in the two

temperate soils (Table 2). In deionized water controls, the $K_{m(app)}$ values were 15 and 6.7 μl liter^{-1} (22 and 9.8 nM in solution) in the hardwood and pine soils, respectively. In the hardwood soil, the values of both K_m and V_{max} were about double the corresponding values in the pine soil, so the pseudo-first-order rate constants (V_{max}/K_m) were similar in the two soils (Table 2). K^+ salts (irrespective of the anions) either decreased or had no effect on $K_{m(app)}$ values compared to water controls, whereas NH_4^+ salts always increased the $K_{m(app)}$. In contrast, $V_{max(app)}$ values decreased similarly in the presence of both K^+ and NH_4^+ salts. Compared to K^+ salts, however, NH_4^+ salts increased $K_{m(app)}$ but had no effect on $V_{max(app)}$.

DISCUSSION

Often, soil CH_4 oxidation at near-atmospheric CH_4 concentrations follows first-order reaction kinetics (3, 39, 46), as was the case in the birch taiga soil in this study (Fig. 1a). In fine-textured soils, first-order kinetics at lower CH_4 concentrations may result from restricted gas diffusion from the atmosphere into the soil, a purely first-order process (14, 37, 45). The birch taiga soil studied is a fine silt soil and therefore strongly limits gas diffusion from the atmosphere to the CH_4 oxidizers (14, 37), thus possibly increasing the $K_{m(app)}$ and creating a problem for studying low-concentration CH_4 oxidation kinetics in this soil (Fig. 3). By contrast, the two temperate forest soils studied have a coarse sandy texture, which enhances CH_4 diffusion, allowing uptake kinetics to reflect enzyme activity more closely and permitting standard kinetic analyses of NH_4^+ and salt inhibition of CH_4 oxidation at near-atmospheric CH_4 concentrations. The maximum CH_4 concentration used in our experiments (15 μl liter^{-1}) was similar to the $K_{m(app)}$ values in the temperate forest soils. The regression curves resulting from the kinetic analyses provided very good fits to the actual data (generally, $R^2 > 0.98$) (Fig. 1; Table 2), indicating that the kinetic models which we used accurately described the process as measured in this study.

Although there have been numerous reports of steady-state kinetic constants for soil CH_4 oxidation (2–4, 15, 36, 46, 47), only one previous study reported kinetic parameters for NH_4^+ inhibition (15). The soil studied previously was an agricultural humisol with an organic matter content of ~70% and demonstrable methanogenic activity (15, 16). These conditions probably supported a much different CH_4 oxidizer community than the community expected in upland mineral soils that lack an endogenous CH_4 source. Indeed, the $K_{m(app)}$ values for our temperate forest soils (Table 2) were substantially lower than the $K_{m(app)}$ values reported in the previous study (15), suggesting that there were physiological differences in the CH_4 oxidizer communities in the upland mineral soils and the agricultural humisol. In the present study we focused specifically on high-affinity CH_4 oxidation in three non-CH_4-producing upland soils from two North American biomes, subarctic taiga forest and northeastern temperate forest.

Salt effects. Interpreting inhibition mechanisms based on kinetic parameters in a system that is as biologically and chemically complex as soil requires careful consideration of how ions added to the system may affect the process of interest, both directly and indirectly (15, 28). All of the ions used in this study potentially could affect CH_4 oxidation in three basic ways. First, they could change the soil osmotic potential and impose water stress on the microbial community (18, 40); second, they could affect ion exchange, thereby altering NH_4^+ availability (28); and third, they could affect the CH_4 oxidizers directly in a number of ways (11, 15, 21). Any of these factors could alter CH_4 oxidation rates and kinetics and thus affect the interpretation of the specific NH_4^+ inhibition mechanism.

With the salt additions used in this study, the water potential in the birch taiga soil was approximately −0.2 MPa, which is the optimum water potential for atmospheric CH_4 oxidation in a wide variety of upland soils (18, 40). Because the birch taiga soil had the lowest water-holding capacity of the soils used in this study (i.e., the lowest water/salt ratio [Table 1]), its osmotic potential should have been the most sensitive to the salt additions. Thus, salt-related inhibition of atmospheric CH_4 oxidation in this study did not appear to be related to water stress.

TABLE 2. CH_4 oxidation kinetics in three upland forest soils

Soil	Treatment	$K_{m(app)}$ (μl liter^{-1})[a]	$V_{max(app)}$ (pmol g^{-1} h^{-1})	First-order rate constant (h^{-1})[b]		% Inhibition compared to[c]:		Implied inhibition mechanism	Curve fit (R^2)[d]
				True	Pseudo	H_2O	K^+		
Birch taiga	H_2O			0.00711					0.994
	K_2SO_4			0.00653		8.2		?	0.996
	$(NH_4)_2SO_4$			0.00526		26*	19*	?	0.992
	KCl			0.00078		89*		?	0.772
	NH_4Cl			0.00072		90*	7.7	?	0.771
Temperate hardwood	H_2O	15.2 (1.3)	5,807 (329)		0.705				0.998
	K_2SO_4	9.8 (0.4)	2,662 (584)		0.510	28*		Uncompetitive	0.999
	$(NH_4)_2SO_4$	22.4 (1.8)	2,870 (157)		0.235	67*	54*	Mixed competitive	0.998
	KCl	7.2 (0.6)	786 (30)		0.200	72*		Uncompetitive	0.993
	NH_4Cl	25.6 (4.9)	1,174 (155)		0.085	88*	58*	Mixed competitive	0.991
Temperate pine	H_2O	6.7 (0.9)	2,594 (160)		0.715				0.984
	K_2SO_4	6.1 (0.9)	1,591 (98)		0.485	32*		Noncompetitive	0.978
	$(NH_4)_2SO_4$	9.8 (1.4)	1,699 (123)		0.320	55*	34*	Mixed competitive	0.986
	KCl	8.8 (4.0)	573 (123)		0.120	83*		Noncompetitive	0.855
	NH_4Cl	10.9 (7.2)	515 (176)		0.085	88	29	Mixed competitive	0.769

[a] The values in parentheses are standard errors.
[b] True first-order rate constants were calculated by linear regression of CH_4 oxidation rates against midpoint CH_4 concentrations. Pseudo-first-order rate constants were calculated by determining V_{max}/K_m after K_m values were converted to picomoles of CH_4 per bottle.
[c] An asterisk indicates that the treatment value was statistically different from the corresponding control value ($P \leq 0.05$).
[d] The regression coefficients are linear for the birch taiga soil and nonlinear (Michaelis-Menten curve fit) for the two temperate soils.

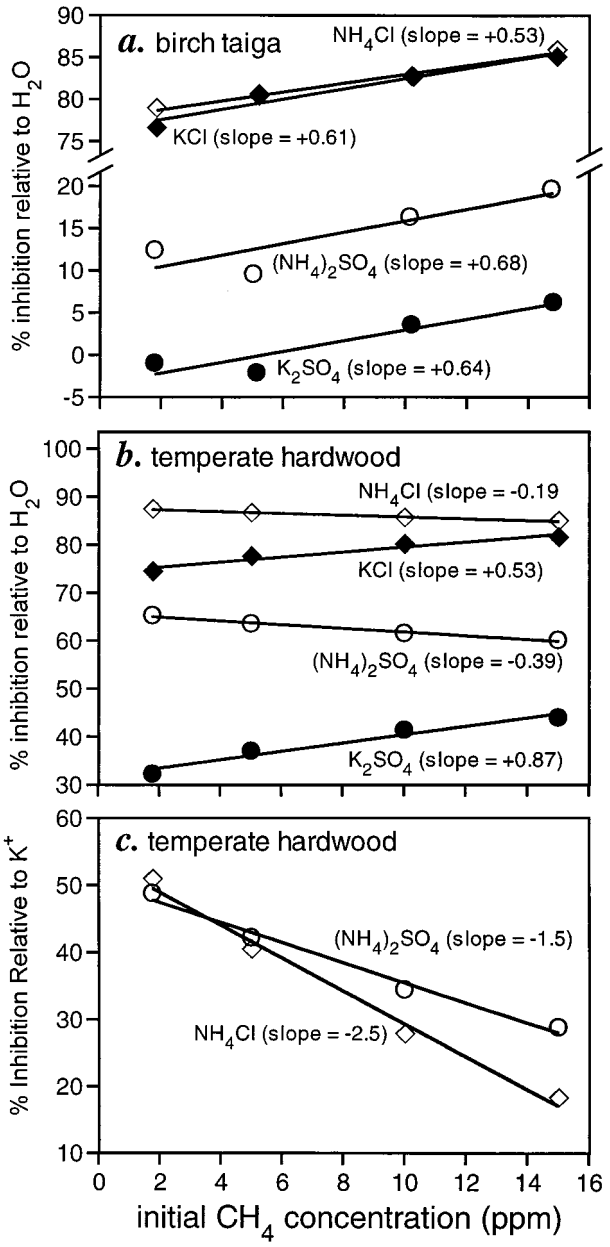

FIG. 2. Effect of CH_4 concentration on inhibition of CH_4 oxidation. General salt inhibition compared to water controls in the birch taiga soil (a) and in the temperate hardwood soil (b). (c) Specific NH_4^+ inhibition compared to K^+ controls in the temperate hardwood soil.

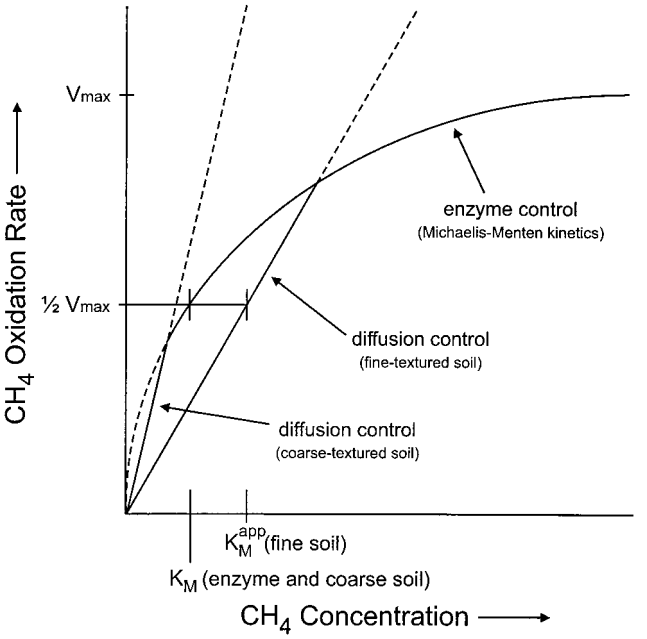

FIG. 3. Potential effect of soil texture on CH_4 oxidation kinetics in soil. Because gas transport by diffusion is purely first order, a fine-textured soil may exhibit first-order kinetics at lower CH_4 concentrations, whereas a coarse-textured soil containing the same CH_4-oxidizing enzyme or a similar enzyme may exhibit Michaelis-Menten kinetics at the same concentrations.

It is unlikely that K^+ salts indirectly produced the inhibition observed in this study by desorbing NH_4^+ from cation exchange sites, as proposed elsewhere (28). This mechanism requires that an untreated soil contain sufficient exchangeable NH_4^+ to account for the inhibition observed with nonammoniacal salts, yet the soils we studied had very low concentrations of exchangeable NH_4^+ relative to our NH_4^+ additions (Table 1). Cl^- salts consistently inhibit soil CH_4 consumption to a far greater extent than do SO_4^{2-} salts (28; this study). King and Schnell (28) attributed this phenomenon to greater NH_4^+ adsorption to cation exchange sites in the presence of SO_4^{2-} than in the presence of Cl^-. In the present study, however, it was impossible for the KCl treatments to produce free NH_4^+ concentrations approaching those of the $(NH_4)_2SO_4$ treatments, because we added 1 to 3 orders of magnitude more NH_4^+ than was potentially available in the untreated soils (Table 1). Even so, KCl inhibition was far greater than $(NH_4)_2SO_4$ inhibition in all three soils (Fig. 1; Table 2). KCl and NH_4Cl produced similar levels of inhibition in each of the soils, despite the fact that the NH_4Cl treatments necessarily resulted in much higher free NH_4^+ concentrations. Similarly, the results of King and Schnell show that NaCl caused inhibition equal to or greater than the inhibition that equinormal NH_4Cl caused in another temperate forest soil (28). Again, this result could not have been dependent on NH_4^+ concentrations. Hence, desorption of endogenous NH_4^+ cannot account for the extremely inhibitory effects of Cl^- salts in a variety of soils, and it is clear that Cl^- salts should be avoided in NH_4^+ inhibition studies, unless it can be demonstrated that Cl^- is not toxic to CH_4 oxidizers in a particular soil.

Unlike KCl, K_2SO_4 inhibited CH_4 oxidation less than $(NH_4)_2SO_4$ inhibited CH_4 oxidation, raising the possibility that there is indirect inhibition by cation exchange when SO_4^{2-} salts are used. Compared to water, K_2SO_4 inhibition was 32 to 58% of $(NH_4)_2SO_4$ inhibition in the three soils (Table 2). Assuming that the desorption of endogenous soil NH_4^+ was 100%, which is unlikely, the K_2SO_4 treatments would have produced maximum NH_4^+ concentrations that were between ~0.1 and 10% of the amount added in the $(NH_4)_2SO_4$ treatment (Table 1). Hence, compared to the $(NH_4)_2SO_4$ treatments, the ratios of relative inhibition to potential NH_4^+ concentration obtained with the K_2SO_4 treatments seem unlikely. More importantly, as discussed below, steady-state kinetic parameters indicate that K_2SO_4 and $(NH_4)_2SO_4$ inhibited CH_4 oxidation via different physiological mechanisms, which would not be the case if K^+ acted indirectly via NH_4^+ desorption.

The ubiquity of NH_4^+-independent salt effects and the variety of salts that induce similar responses suggest that a fun-

damental physiological process is involved. Roslev et al. (36) found that high-affinity CH_4 oxidizers in a temperate forest soil efficiently incorporated $^{14}CH_4$-C into biomass. Adding NH_4Cl to the soil not only decreased CH_4 oxidation rates but also reduced the C assimilation efficiency and dramatically increased the proportion of ^{14}C oxidized to CO_2. It is impossible to know whether this response was to NH_4^+ or Cl^- or both, as the experiments did not include parallel salt controls. However, because we found that Cl^- overwhelmingly dominated NH_4Cl inhibition in all of our soils, the response that Roslev et al. observed may also have been predominantly due to Cl^-. Killham (25) reported that NaCl additions had the same effect on microbial assimilation and respiration of [^{14}C]glucose in soil and found that an increase in the ratio of respired C to assimilated C was a sensitive index of physiological stress within a soil heterotroph community. Shifts in the ratio were attributed to an increase in the maintenance energy required for the cells to cope with the imposed stress. If active transport of ions out of the cell or some other energy-intensive coping strategy were required by energy-limited CH_4 oxidizers exposed to a salt, then cellular reductant might be diverted to this process, making less reductant available for growth and potentially to the CH_4-oxidizing enzyme, thus decreasing the CH_4 oxidation rates. This scenario is plausible for an extremely energy-limited population and is reconcilable with the inhibition kinetics reported here, as diverting reductant away from the CH_4-oxidizing enzymes should reduce the catalytic efficiency of the extant enzyme pool, thereby potentially altering $K_{m(app)}$ and $V_{max(app)}$ as described below. Gulledge et al. (17) observed an apparent growth response of the atmospheric CH_4 oxidizer community in samples obtained from depths of 20 to 40 cm in another forest soil. The in situ CH_4 concentrations at depths below 20 cm were chronically <0.5 μl liter^{-1}. After 14 days of exposure to ambient atmospheric CH_4 in the laboratory, the CH_4 consumption rates in water-treated samples increased severalfold compared to the rates measured after only 5 days of exposure. In K_2SO_4-treated samples a less pronounced increase occurred, and in $(NH_4)_2SO_4$-treated samples no increase occurred, suggesting that the effects of NH_4^+ and salt were synergistic. These results also are consistent with a cellular stress response by an energy-limited population and may illustrate why atmospheric CH_4 oxidizers have a limited capacity to recover from soil fertilization (32, 33, 39).

If salts generally inhibit soil CH_4 oxidation by an NH_4^+-independent mechanism, then it seems appropriate to quantify specific NH_4^+ inhibition based on a parallel salt control rather than a deionized water control. This approach has been challenged by the view that other cations may have unique inhibition mechanisms that make them ineffective as experimental controls (28). Although this hypothesis is plausible, no differential toxicity of potential control cations, such as Na^+ and K^+, has been reported for soil CH_4 oxidation. King and Schnell (28) found that KCl inhibited CH_4 uptake by pure cultures of *Methylosinus trichosporium* more than did NaCl, but they observed no difference in soil CH_4 consumption in the presence of these two salts. Similarly, we observed no difference in the effects of K_2SO_4 and Na_2SO_4 in the birch taiga soil in the present study (the temperate soils were not tested with Na^+). Moreover, it is equally plausible that similar cations, such as NH_4^+, K^+, and Na^+, exert equivalent nonspecific effects that, in conjunction with counteranion effects, account for the nonammoniacal inhibition observed with salts in general. Since K^+ and Na^+ salts inhibit soil CH_4 oxidation to the same extent (28; this study), this hypothesis appears to be sound. Our view, therefore, is that parallel salt controls must be employed when NH_4^+ inhibition is examined, because there is no other way to account for the nonammoniacal effects that salts clearly have on soil CH_4 consumption. In some cases, salt effects can be substantial compared to specific NH_4^+ inhibition and therefore probably interfere with kinetic analysis of the NH_4^+ inhibition mechanism. In the present study we used both deionized water and nonammoniacal salt controls in order to examine the relative efficacies of the two approaches for elucidating the mechanism of NH_4^+ inhibition.

Specific NH_4^+ inhibition. Determining the physiological mechanism of specific, immediate NH_4^+ inhibition has proven to be difficult (6, 15, 17, 26–28). Dunfield and Knowles (15) demonstrated that NH_4^+ inhibited CH_4 oxidation by enzymatic substrate competition in an agricultural humisol assayed at high CH_4 concentrations. The kinetics varied between samples, however, indicating that an additional mechanism may have been involved. King and Schnell (27, 39) examined NH_4Cl inhibition at low CH_4 concentrations and found that relative inhibition increased with CH_4 concentration. They concluded that this phenomenon resulted from the fortuitous oxidation of NH_4^+ to toxic NO_2^- or NH_2OH, which in turn reduced the activity of the methanotroph population (39). They did not examine Michaelis constants or comparable kinetic parameters. We observed similar increases in inhibition with increasing CH_4 concentrations in all three of the soils we examined. In the taiga soil, this phenomenon occurred with nonammoniacal salts as well as NH_4^+ salts, and the slope of the increase was not affected by the NH_4^+ concentration (Fig. 2a). In the temperate soils, K^+ salts caused inhibition to increase, whereas NH_4^+ salts caused inhibition to decrease as the CH_4 concentration increased (Fig. 2b and c). The same pattern occurred whether Cl^- or SO_4^{2-} salts were added, indicating that it was not specific to a particular counterion (Fig. 2a and b). These results indicate that the increase in inhibition did not result from NH_4^+ or its by-products. Thus, although NO_2^- undoubtedly inhibits atmospheric CH_4 oxidation when it is added directly to soil (19, 26), an increase in NH_4^+ salt inhibition when the CH_4 concentration increases more likely results from a general salt effect than from by-products of fortuitous NH_4^+ oxidation.

A net increase in inhibition with an increase in the CH_4 concentration in response to NH_4^+ salts may actually indicate that specific NH_4^+ inhibition is weak or absent. For instance, in the birch taiga soil, in which NH_4^+ inhibition was relatively weak (Table 2), both NH_4^+ and K^+ salts caused similar increases in inhibition as the CH_4 concentration increased (Fig. 2a). However, in the temperate hardwood soil, in which NH_4^+ inhibition was relatively strong (Table 2), only K^+ salts caused inhibition to increase, whereas NH_4^+ salts caused inhibition to decrease as the concentration of CH_4 increased (Fig. 2b). Specific NH_4^+ inhibition, isolated by using K^+ salts as controls, declined precipitously as the CH_4 concentration increased (Fig. 2c). Hence, salts generally caused increases in inhibition, whereas NH_4^+ caused decreases in inhibition as the CH_4 concentration increased, indicating that there are separate inhibition mechanisms for NH_4^+ specifically and salts generally. In our soils, the relative strengths of these two mechanisms were apparent from the slopes of the plots of $(NH_4)_2SO_4$ inhibition (relative to deionized water) versus CH_4 concentration; a positive slope indicated a stronger salt effect, whereas a negative slope indicated a stronger NH_4^+ effect.

The soils which we examined were relatively acidic (pH ~3.5 to 4.5). Because NH_3, rather than NH_4^+, is probably the competitive inhibitor of CH_4 oxidation, salt effects may be more prevalent in acidic soils, whereas competitive inhibition may be relatively more important in neutral to alkaline soils, such as the agricultural humisol investigated by Dunfield and Knowles

(15). Despite the intuitive appeal of this hypothesis, there is no obvious relationship between pH and the degree of NH_4^+ inhibition in soils with different pH values, suggesting that other cross-site variables are generally more important (17). Moreover, it is clear from the results obtained with the temperate hardwood soil, in which NH_4^+ accounted for 58% of the total inhibition, that NH_4^+ inhibition can be dominant in acidic soils. Perhaps the intracellular pH, which should be near neutral regardless of the soil pH, is the relevant control on NH_3/NH_4^+ ratios at the enzyme level.

Compared to the K_m in the water controls, the $K_{m(app)}$ either decreased (hardwood soil) or remained unchanged (pine soil) when K^+ salts were added, but it always increased when NH_4^+ salts were added (Table 2). Again, this pattern supports the hypothesis that there are different inhibition mechanisms for NH_4^+ and salts in general and also eliminates the possibility that K^+ salts acted indirectly by desorbing soil-bound NH_4^+ into solution, as concluded previously for another temperate forest soil (28). If K^+ ions acted indirectly via NH_4^+, then K^+ and NH_4^+ salts should have produced similar inhibition kinetics, yet they had different effects on $K_{m(app)}$ compared to deionized water. In contrast to $K_{m(app)}$, $V_{max(app)}$ decreased in response to both K^+ and NH_4^+ salts (Table 2). Thus, the kinetic constants suggest that there is a partial mixed-type inhibition for NH_4^+ salts, with both a competitive component (increasing K_m) and a noncompetitive or uncompetitive component (decreasing V_{max}) that is independent of NH_4^+ (41) (Table 2). If general salt effects account for the additional inhibition, then using K_2SO_4 as a control rather than deionized water should isolate the specific NH_4^+ effect. Indeed, compared to K^+ salts, NH_4^+ salts caused $K_{m(app)}$ to increase substantially, whereas they had no effect on $V_{max(app)}$ (Table 2). The Cl^--salt pair produced the same kinetic pattern as the SO_4^{2-} pair despite the greater inhibition by Cl^-. These consistent results strongly indicate that NH_4^+ inhibited atmospheric CH_4 oxidation in the two temperate forest soils via simple enzyme substrate competition.

Summary and conclusions. Our approach using K^+ salts as controls, and the resulting interpretation, provided a plausible NH_4^+ inhibition mechanism that is consistent with the data presented here and can also account for the contrasting results of previous studies (15, 27, 39). Whereas Dunfield and Knowles (15) observed competitive inhibition kinetics in their agricultural humisol, Schnell and King observed increasing inhibition with increasing CH_4 concentrations in a temperate forest soil, a result that, by itself, is inconsistent with competitive inhibition. Both phenomena occurred simultaneously in our temperate forest soils and could be explained by a mixed-type inhibition resulting from at least two independent mechanisms, enzymatic substrate competition by NH_4^+ and one or more noncompetitive or uncompetitive mechanisms common to salts in general. Although the inhibition mechanism in the birch taiga soil could not be determined directly because it displayed first-order kinetics (Fig. 1a), the relative inhibition pattern for the various treatments was consistent with the patterns obtained with the two temperate soils, so that all three soils may have shared the same mechanisms. Moreover, it is notable that despite very different soil characteristics, we found essentially the same inhibition mechanism that Dunfield and Knowles (15) found in an agricultural humisol. This convergence of physiological responses in ecologically diverse environments suggests that enzymatic substrate competition is an important NH_4^+ inhibition mechanism in a wide variety of soils.

Although the results readily explain immediate inhibition of soil CH_4 oxidation, delayed inhibition, which has been observed in both field and laboratory studies (17), remains enigmatic. Delayed inhibition probably results from shrinkage of the CH_4 oxidizer population over time rather than from decreases in the specific activities of individual CH_4 oxidizers (17). NH_4^+ and salt effects may act synergistically to impose whole-cell stress that increases maintenance energy requirements, thereby diverting reductant from growth, even if sufficient reductant for the CH_4-oxidizing enzyme remains available. This scenario might diminish a population's ability to replace dying biomass, yet might not slow the oxidation of CH_4 until the population begins to shrink, resulting in a delayed inhibition response (17). Hence, multiple physiological mechanisms may contribute synergistically to both immediate and delayed NH_4^+ fertilizer inhibition of atmospheric CH_4 consumption in soil. Moreover, nonammoniacal salts in the environment, especially KCl and NaCl (both of which are used heavily in agriculture and industry), may be as problematic as NH_4^+ fertilizers for soil CH_4 consumption.

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